PATENT

Docket No. 19603/267 (CRF D-933)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Barany, et al.	Examiner: E. Grimes
Serial No:	08/462,221	Art Unit: 1814
Filed:	June 5, 1995	
For:	THERMOSTABLE LIGASE-MEDIATED DNA AMPLIFICATION SYSTEM FOR THE DETECTION OF GENETIC DISEASE	

DECLARATION OF FRANCIS BARANY UNDER 37 CFR § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, FRANCIS BARANY, declare:

1. I received a B.A. in Chemistry from the University of Illinois at Chicago Circle, Chicago, Illinois in 1976 and a Ph.D. degree in Microbiology from the Rockefeller University, New York, New York in 1981. I conducted postdoctoral work from 1981 to 1982 in microbiology at the Rockefeller University, New York, New York and from 1982 to 1985 in molecular biology at the Johns Hopkins University School of Medicine, Baltimore, Maryland.

2. I am a Professor, Department of Microbiology, Cornell University Medical College, New York, New York and an Adjunct Professor at the Rockefeller University, New York, New York.

3. I am a co-inventor of the above-identified patent application.

4. In support of the above-identified patent application, I supervised studies on the fidelity of DNA ligase from the thermophilic bacteria, *Thermus thermophilous* ("*Tth* DNA ligase"), to evaluate the ability of the ligase chain reaction process to detect genetic diseases. More particularly, a fluorescent assay for quantitative

analysis of the fidelity of *Tth* DNA ligase against every possible mismatch on both the 3' and 5' side of a nick was conducted.

5. Prior to performing the fidelity assay, the Tth DNA Ligase was purified as described in Barany, F., et al., "Cloning, Overexpression, and Nucleotide Sequence of a Thermostable DNA Ligase Gene," Gene. 109:1-11 (1991). Nicked DNA duplex substrates were formed by annealing two oligonucleotides (the discriminating oligonucleotide and the fluorescently labeled oligonucleotide) on top of a longer complementary oligonucleotide, the template.

6. As shown in Figure 1 (attached hereto as Exhibit 1), one of the four long oligonucleotides GLg, ALg, TLg or CLg (shown in the bottom panel) was used as the template strand, which vary at the underlined base. The upper and middle panels represent the formation of nicked DNA duplex using one of the template strands, ALg, as an example. Shown in the upper panel are 4 different nicked DNA substrates formed by annealing the common fluorescently-labeled oligo, com5F, and one of the discriminating oligos (RP5'A, RP5'C, RP5'G, or RP5'T) to the template strand, ALg. In the middle panel, 4 different nicked DNA substrates are formed by annealing the fluorescently-labeled oligo, com3F, and one of the discriminating oligos (LP3'A, LP3'C, LP3'G, or LP3'T) to the template strand, ALg. A matrix of nicked duplexes is thus formed with all possible combinations of match and mismatch base pairing on the 3' and the 5' side of the nick, when ALg is replaced by the GLg, TLg, or CLg template strands. Products formed by ligation to the common fluorescently-labeled primers can be discriminated by size on denaturing polyacrylamide gel due to the incorporation of different "A" tail lengths.

7. Sequences of these primers (shown in Table I) were derived from that of human eukaryotic protein synthesis initiation factor eIF-4E. Rychlik, W., et al., "Amino Acid Sequence of the mRNA Cap-binding Protein from Human Tissues," Proc. Natl. Acad. Sci. USA. 84: 945-949 (1987).

Table I: Sequences of oligonucleotides used for the fluorescent assay of the fidelity of *Tth* DNA ligase.

Primer name	Sequence
RP5'A	5' AA GTT GTC ATA GTT TGA TCC TCT AGT CTG GG AAA AAA 3'
RP5'C	5' CA GTT GTC ATA GTT TGA TCC TCT AGT CTG GG AAA A 3'
RP5'G	5' GA GTT GTC ATA GTT TGA TCC TCT AGT CTG GG AA 3'
RP5'T	5' TA GTT GTC ATA GTT TGA TCC TCT AGT CTG GG 3'
Com 5F	5' F-CCC TGT TCC AGC GTC TGC GGT GTT GCG T 3'
LP3'A	5' AAA AAA CCC TGT TCC AGC GTC TGC GGT GTT GCG TA 3'
LP3'C	5' A AAA CCC TGT TCC AGC GTC TGC GGT GTT GCG TC 3'
LP3'G	5' AA CCC TGT TCC AGC GTC TGC GGT GTT GCG TG 3'
LP3'T	5' CCC TGT TCC AGC GTC TGC GGT GTT GCG TT 3'
Com 3F	5' A GTT GTC ATA GTT TGA TCC TCT AGT CTG GG-F 3'
ALg	5' CCC AGA CTA GAG GAT CAA ACT ATG ACA ACT AAC GCA ACA CCG CAG ACG CTG GAA CAG GG 3'
CLg	5' CCC AGA CTA GAG GAT CAA ACT ATG ACA ACT CAC GCA ACA CCG CAG ACG CTG GAA CAG GG 3'
GLg	5' CCC AGA CTA GAG GAT CAA ACT ATG ACA ACT GAC GCA ACA CCG CAG ACG CTG GAA CAG GG 3'
TLg	5' CCC AGA CTA GAG GAT CAA ACT ATG ACA ACT TAC GCA ACA CCG CAG ACG CTG GAA CAG GG 3'

A random DNA sequence from an eukaryotic source was chosen to avoid any false signal arising from possible bacterial DNA contamination in *Tth* DNA ligase preparation. The melting temperature of primers were predicted using the nearest neighbor thermodynamic method of Breslauer, K.J. et al., "Predicting DNA Duplex Stability From the Base Sequence," Proc. Natl. Acad. Sci. USA, 83:3746-3750 (1986). The OLIGO 4.0 program from National Biosciences Inc., Plymouth, MN was used to rule out possible hairpin structure or repetitive sequences. The templates and detecting oligonucleotides for this assay have been designed such that their melting temperatures are sufficiently higher than the temperature used in the assay (i.e., 65°C) to minimize the effect of the melting temperature of primers on ligation efficiency.

8. All oligonucleotide primers were synthesized using reagents and a 394 automated DNA synthesizer from Applied Biosystem Division of Perkin-Elmer

Corporation, Foster City, CA. Synthesis of oligonucleotides with a fluorescent dye, 6-FAM (i.e., 6-carboxy Fluorescin), attached at the 5' end was done using 6-FAM Amidites from Applied Biosystem Division of Perkin-Elmer Corporation. The oligonucleotide with a 3' FAM was made by using a 3'-Amino-modifier C3-CPG column from Glen Research (Sterling, VA) for the initial DNA synthesis, and the FAM group was then attached through the 3'-amino group using the NHS-FAM (i.e., the N-hydroxyl Succinimide ester of FAM) from the Applied Biosystem Division of Perkin-Elmer Corporation. All oligonucleotides used in this study were purified by polyacrylamide gel electrophoresis and recovery of DNA from gel slices using C-18 Sep-Pak Cartridges from the Waters Division of Millipore.

9. Oligonucleotide primers, RP5'A, RP5'C, RP5'G, RP5'T, and Com3'F, were 5'-phosphorylated in a 25 µl reaction solution containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 1 mM ATP, 1 nmole of gel-purified oligonucleotides, and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) at 37°C for 45 minutes. The reaction was quenched by adding 0.5 µl of 0.5 M EDTA, and the kinase was heat-inactivated by incubation at 64°C for 20 minutes. The phosphorylated oligonucleotides were stored at -20°C in 5 µl aliquots before use.

10. Each reaction was performed in 40 µl of buffer containing 20 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 100 mM KCl; 10 mM DTT; 1 mM NAD^+; and 12.5 nM (500 fmoles) of nicked duplex substrates. DNA primers were denatured (94°C for 2 min.) reannealed (65°C for 2 min.), and ligations initiated by the addition of 0.125 nM (5 fmoles) *Tth* DNA ligase and carried out at 65°C. Five µl aliquots were removed at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 23 hr, and mixed with 18 µl of a stop solution (83% formamide, 8.3 mM EDTA, and 0.17% Blue Dextran). To 5 µl of this mixture, 0.5 µl of Rox-1000, a fluorescently labeled in-lane size standard (Applied Biosystem Division of Perkin-Elmer) was added. Samples were denatured at 93°C for 2 min, chilled rapidly on ice prior to loading on an 8 M urea-10% polyacrylamide gel, and electrophoresed at 1400 V (constant voltage) on a model 373A automated DNA Sequencer from Applied Biosystem Division of Perkin-Elmer Corporation, Foster City, CA. Electrophoresis conditions were modified as suggested by the manufacturer. The gel was polymerized in 1.2 x TBE (54 mM Tris-Borate and 1.2 mM EDTA, pH 8.0) and was pre-run before loading samples in a running buffer of 0.6 x TBE (27 mM Tris-Borate and 0.6 mM EDTA, pH 8.0) for 30 min with an

electrode polarity opposite to the normal run with samples. After pre-run and sample-loading, the gel was run in 0.6 x TBE in the normal top to bottom direction for 2.5 hrs. Fluorescently labeled ligation products were analyzed and quantitated using Genescan 672 version 1.2 software (Applied Biosystem Division of Perkin-Elmer Corporation, Foster City, CA), and the results were plotted using DeltaGraph Pro3 software (DeltaPoint, Inc., Monterey, CA). Genescan 672 software provided analyzed data in the form of a gel image and a table, continuing the peak height and peak area of each peak in each lane. Typically, two bands were seen in each lane. The lower one was the free fluorescent common oligonucleotide, the upper one was ~~the upper strand of~~ the ligation product. The product yield in percentage was calculated as product over total initial substrate times 100. The amount of product was calculated as the peak area of the appropriate ligation product. The amount of initial substrates were calculated by adding the peak area of the product peak to that of the free fluorescent oligonucleotide peak. Results were plotted using DeltaGraph Pro3 software (DeltaPoint, Inc., Monterey, CA) with Time on the X-axis and yield on the Y-axis.

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11. Figure 2 and Table II summarize the results of the fidelity of nick closure by *Tth* DNA ligase on the 3' side of the nick.

Table II: Ligation yield generated by *Tth* DNA ligase with different DNA substrates containing different baseparing on the 3' side of the nick.

Base-pairing	0 hour	2 hour	4 hour	6 hour	8 hour	23 hour
A-A	0%	0%	0%	0%	0%	0%
A-T	0%	85.3%	88.9%	90.2%	92%	93.2%
A-G	0%	0%	0%	0%	0%	0%
A-C	0%	0%	0%	0%	0%	1.1%
C-A	0%	0%	0%	1.3%	1.8%	2%
C-T	0%	0%	0%	0%	0%	0%
C-G	0%	82%	88%	90%	96.5%	93.3%
C-C	0%	0%	0%	0%	0%	0%
G-A	0%	0%	0%	0%	0%	0%
G-T	0%	2.5%	3.8%	5.46%	7.5%	11%
G-G	0%	0%	0%	0%	0%	0%
G-C	0%	82.6%	87.4%	87.4%	88.9%	91.3%
T-A	0%	83.8%	86.5%	88.4%	89%	92.6%
T-T	0%	0%	0%	0%	0%	0%
T-G	0%	2.2%	3.8%	4.45%	8.8%	13.4%
T-C	0%	0%	0%	0%	0%	0%

Litigation reactions were carried out as described above. Specific base-pairing between the discriminating oligonucleotide and the template on the 3' side of the nick in each substrate are shown in the first column. Aliquots were removed from the reaction mixture at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 23 hr. Ligation yield was calculated by dividing the amount of product by the amount of total initial substrates.

Each panel in Figure 2 (attached hereto as Exhibit 2) shows the yield of product formed with the same discriminating oligonucleotide and fluorescently labeled oligonucleotide annealed to four template strands which differ by a single base. Therefore, each substrate differs by only one base on the template strand. The yield of all mismatches was less than 15% even after 23 hours of incubation, while the yields of wildtype enzyme reached near 90% within two hours. In other experiments (not shown) with an A-T match, yields of product us wildtype enzyme were 79% after 15 minutes, 85% after 30 minutes, 88% after 1 hour, and 92% after 2 hours. With a C-G match, such yields were 66% after 15 minutes, 77% after 30 minutes, 81% after 1 hour, and 90% after 2 hours. Of all 12 mismatches tested on the 3' side of the nick, T-G and G-T mismatches were the only mismatches which accumulated a yield of about 15% over a 23 hour incubation period in a linear fashion,

while the yields for the rest of the mismatches were less than 5%. Therefore, T-G and G-T mismatches were the least discriminated mismatches by *Tth* DNA ligase.

12. When the mismatches were located at the 5' side of the nick, the fidelity pattern of the *Tth* DNA ligase was quite different (Figure 3 (Attached hereto as Exhibit 3) and Table III).

Table III: Ligation yield generated by *Tth* DNA ligase with different DNA substrates containing different baseparing on the 5' side of the nick.

Base-pairing	0 hour	2 hour	4 hour	6 hour	8 hour	23 hour
A-A	0%	3.2%	4.45%	6.7%	7.45%	23.6%
A-T	0%	79.6%	82.2%	84.2%	85.6%	89.1%
A-G	0%	0%	0%	0%	0%	0%
A-C	0%	2.8%	4.4%	7.4%	8.7%	22.8%
C-A	0%	7.3%	13.5%	19%	23.7%	38.2%
C-T	0%	0%	1.47%	2%	2.7%	6.5%
C-G	0%	78%	83.5%	84.8%	84.2%	89.5%
C-C	0%	0%	0%	0%	0%	0.86%
G-A	0%	1.8%	4.4%	6.8%	10.5%	20%
G-T	0%	2.1%	5.34%	7.5%	11.7%	20.5%
G-G	0%	0%	0%	0%	0%	1.03%
G-C	0%	76.2%	80.9%	84.1%	86.3%	87.2%
T-A	0%	78.7%	83.8%	84.5%	86.4%	87.8%
T-T	0%	14.3%	22.7%	30%	38.6%	44.5%
T-G	0%	8.9%	14.3%	20.8%	30.4%	44.9%
T-C	0%	0%	0%	0%	0%	1.1%

Litigation reactions were carried out as described above. Specific base-pairing between the discriminating oligonucleotide and the template on the 5' side of the nick in each substrate are shown in the first column. Aliquots were removed from the reaction mixture at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 23 hr. Ligation yield was calculated by dividing the amount of product by the amount of total initial substrates.

While the performance of the enzyme to perfect matches and some mismatches (i.e., A-G, C-C, G-G, and T-C) did not change, the discrimination against the rest of mismatches were all increased. Ligation of mismatches A-C, A-A, C-A, G-A, and T-T, which were barely detected after 23 hours of incubation when placed at the 3' side of the nick, became quite significant when placed at the 5' side of the nick. The fidelity results of nick-closure

by *Tth* DNA ligase indicate that the *Tth* DNA ligase discriminates mismatches at the 3' side of the nick much more efficiently than to those placed at the 5' side of the nick. Although T-G and G-T mismatches were less efficiently discriminated by *Tth* DNA ligase compared to other mismatches at 3' side of the nick, the amount of products formed within ~~one~~ two hours with these mismatches was only less than 2% (See Figure 2). The internal stability of discriminating oligonucleotides on both the 3' and the 5' sides of the nick was analyzed using Oligo 4.0 program. This program was originally designed for the analysis of PCR primers. In general, a good PCR primer should have high internal stability on the 5' end and slowly decrease to the lowest at the 3' end. The internal stability is calculated as the sum of the free energy of five continuous bases. It was found that the internal stability of bases near the nick on discriminating oligs at the 5' side of the nick was lower than those on discriminating oligs at the 3' side of the nick. Thus, the fidelity of ligation was expected to be better for mismatches at the 5' side of the nick than those at the 3' side of the nick. Therefore, the observed higher fidelity to mismatches on the 3' side of the nick by Tth ligase was unexpected and was not caused by specific sequence content of the discriminating oligos, but by specific requirements of the nick structure by *Tth* DNA ligase.

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13. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

7/14/95
Date

Dr. Francis Barany
Francis Barany